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MEASURING PROBLEMS IN ESTIMATING THE EXPOSURE TO PASSIVE SMOKING USING THE EXCRETION OF COTININE*

(Passive smoking; urinary cotinine; controlled low exposure levels; inter-intra-laboratory variation; reliability; measuring problems; fractioned sampling)

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SUMMARY

Quality control studies on cotinine measurements following low level environmental tobacco smoke (ETS) exposure are rare. The exposure to ETS was controlled and systematically changed in a series of experiments in a climatic chamber. Healthy nonsmoking volunteers were exposed to ETS simultaneously. The duration and level of exposure varied using high (8, 17 and 25 ppm CO), and low (2 and 5 ppm CO) exposure levels. The variation between radioimmunoassay (RIA) and gas chromatography (GC) was high as was the variation between the results of RIA laboratories. There was also a high within-laboratory-variation. A 1:10 dilution seems to be preferable over a 1:3 dilution. Freezing the urine samples immediately after collection led to the detection of higher cotinine values than freezing the samples 24 h after collection. Highly reliable data for cotinine were obtained when the urine samples were kept frozen immediately after collection and fractionated sampling over 48–72 h was used. Our data show that estimating low-level ETS exposure by measuring urinary cotinine is highly susceptible to uncontrolled variation and errors. Sufficiently reliable estimates of low-level ETS exposure can be made only when fractionated sampling over 48–72 h is used and when the urine samples are kept frozen just after collection.

INTRODUCTION

Cotinine concentrations in urine, plasma, and saliva have been used widely as indicators of recent exposure to ETS. As a metabolite of nicotine cotinine is highly

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Abbreviations: ETS, environmental tobacco smoke; GC, gas chromatography; RIA, radioimmunoassay.

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specific for tobacco smoke. It has been shown that there is a close dose-response relationship between the duration and the level of exposure on one hand, and the urinary cotinine excretion on the other [1-5]. However, the absolute concentrations to be measured can be very low. Following low level of ETS exposure they approach the detection threshold of available laboratory methods and they also show considerable variation. Up to now, with the exception of the paper of Biber et al. [6], we are unaware of studies on quality control regarding the measurement of cotinine following low-level ETS exposure.

A series of experiments in healthy volunteers was carried out in a climatic chamber under strictly controlled conditions. We established a dose-response relationship between experimentally controlled exposure levels and nicotine and cotinine measured in plasma, saliva, and urine. Detailed results have been or will be published elsewhere [2, 14]. In this paper, we report on the results of repeated measurements and the sources of variation which were investigated.

MATERIALS AND METHODS

The exposure sessions lasting from one to six hours took place in a 60 m^3 climatic chamber especially designed for exposure experiments. The conditions of ETS exposure were controlled via a continuous-reading CO-measuring device (Comovarn 100 C, produced by Draeger, Lübeck, F.R.G.). A smoking machine was used to generate the ETS atmosphere according to preset levels ranging from 2 to 30 ppm CO. The mainstream smoke produced was routed outside of the climatised room so that only sidestream smoke polluted the atmosphere. As a check for the validity of CO as a tracer substance for the level of pollution, air samples were taken at various points in time. The mass of captured nicotine was subsequently determined by GC. To ensure that all individuals were subjected to the same level of pollution, for each experiment, they were all exposed simultaneously in a single session in the climatic chamber. In addition, sitting positions were randomly changed at 30-min intervals. Nicotine and cotinine in plasma, saliva and urine were quantified by RIA developed by LANGONE [7] with a modification described by Haley [8]. In this paper, only cotinine values will be discussed.

A series of three experiments was conducted. During the first experiment 10 healthy volunteers were exposed to ETS (25-30 ppm CO) for 3 h [2]. This high exposure level was chosen in an effort to establish some preliminary findings:
- the kinetics of nicotine and cotinine;
- the body fluid most suitable for future measurements;
- a methodological comparison of 2 laboratory techniques (RIA and GC).

In a second experiment 2 × 5 healthy volunteers were exposed to ETS corresponding to 8 and 17 ppm CO. The subjects were randomly allocated to 1 or 2 h of exposure. In this experiment we quantified the total urinary cotinine excretion over 72 h as a function of duration and level of exposure. We also compared the cotinine

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TABLE I

COMPARISON BETW
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measurements within one laboratory as well as between two laboratories [14].

In our third experiment we exposed 9 healthy volunteers over 2 and 6 h at an exposure level corresponding to 2 and 5 ppm CO. These exposure levels reflect realistic conditions. They can and do occur in a variety of settings, e.g., at work, with other people smoking in a small and poorly ventilated office [9]. The cumulative urinary cotinine excretion was quantified over 48 h. We examined the impact of interlaboratory variability on the resulting low-level urinary cotinine concentrations. We investigated the variability of the laboratory methods at the resulting low urinary cotinine levels. We also checked on possibilities to increase the sensitivity of the radio immuno assay method by reducing the dilution of the samples from 1:10 down to 1:3 [14]. Dilution of the original samples is commonly used in RIA techniques to reduce cross reactions with other substances.

RESULTS

Variation between RIA and GC

During our first experiment (25–30 ppm CO over 3 h) RIA was compared to GC (Table I). In saliva and plasma no statistically significant differences between the two methods were found, either for nicotine or for cotinine. The correlation between the two methods was relatively weak with correlation coefficients ranging from -0.43 to 0.85.

In 24-h urine the correlation was much higher ($r = 0.93$) but there were statistically significant differences between the distributions of values measured by RIA vs. GC. The means found by RIA were approx. 30% higher than those of GC. This could indicate either a higher sensitivity or a lower specificity of RIA.

To compare the sensitivity of the two methods, values for saliva and plasma levels were classified as detectable or not detectable (Table II). For plasma, the samples

TABLE I

COMPARISON BETWEEN RADIOIMMUNO ASSAY AND GASCHROMATOGRAPHY FOR MEASURING NICOTINE AND COTININE CONCENTRATIONS IN PLASMA, SALIVA AND URINE

Parameter	Source	n	*P* (sign test)	r
Nicotine	Saliva	8–10	0.51–1.00	0.78–0.85
Plasma		5	0.37	0.22
24-h urine		10	0.04	0.93
Cotinine	Saliva	8	1.00	−0.43
	Plasma	9	0.73	0.71
	Urine	10	0.02	0.93

Statistical analysis was conducted using the sign test.
The linear correlation coefficient r was also calculated (Study I).

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TABLE II

SENSITIVITY OF RADIOIMMUNO ASSAY (RIA) AND GASCHROMATOGRAPHY (GC)

			Saliva Detectable by GC		Plasma Detectable by GC	
			No	Yes	No	Yes
Nicotine	Detectable by RIA	No	0	1	10	0
		Yes	1	8	5	5
Cotinine	Detectable by RIA	No	3	0	4	1
		Yes	6	1	6	9

Only baseline samples or samples with assumed low concentrations of nicotine and cotinine were selected for this part of the analysis. The concentrations measured were dichotomized (detectable/not detectable) (Study I).

collected immediately before and after exposure were pooled. Despite the small number of observations there is a consistent pattern indicating that RIA may be more sensitive (or less specific) than GC. Altogether there were 18 samples with levels detectable by RIA but not detectable by GC. In only 2 samples was the pattern reversed. The high incidence of samples without concentrations detectable by either method was not surprising since two thirds of these samples represent baseline levels.

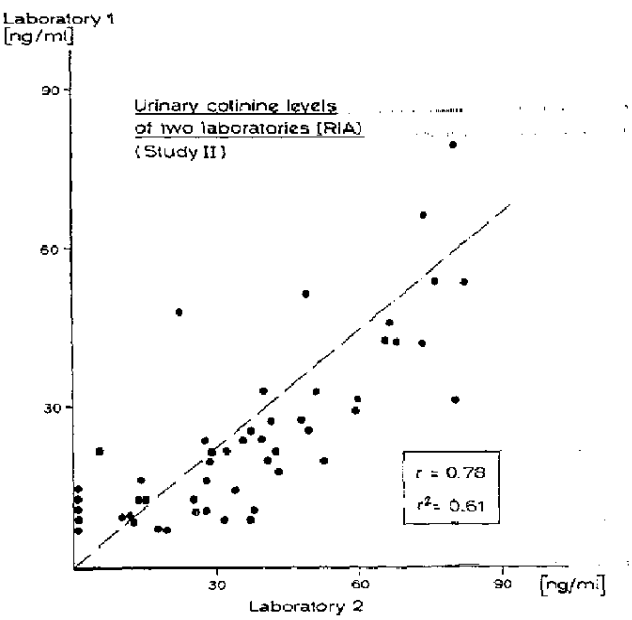

Fig. 1. Variation between laboratories. RIA method according to Langone [7] and Haley [8].

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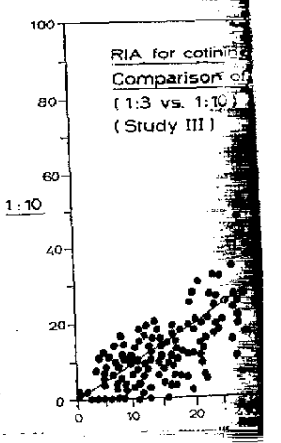

Fig. 2. Variation of
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We found that the highest levels were measured in 24-h urine which integrates the excretion over prolonged periods of time. This allows a better estimate of nicotine uptake than levels in plasma and/or saliva: both of which reflect only a point estimate of nicotine and cotinine.

Variation between laboratories

During our second study (8 and 17 ppm CO over 1 and 2 h) we compared urine cotinine concentrations found by two different laboratories both using a comparable RIA method based upon the Langone procedure (Fig.1). The linear correlation between the values measured by the two institutions is only 0.78. Whether a non-linear model is more appropriate for the data is unclear. Laboratory 1 shows some tendency to systematically measure lower levels of cotinine. But this lab may also have a slightly more sensitive assay at very low levels. There were 5 samples below detection level in laboratory 2, but in laboratory 1 levels between 6 and 14 ng/ml were measured. This indicates that absolute levels found in different studies and measured by different laboratories cannot directly be compared even if they both use the same RIA technique.

Variation by dilution (RIA)

The RIA procedure applied uses a 1:10 dilution of the urine samples. At low or very low levels of cotinine in urine this could mean that the actual concentrations could be diluted below the detection level of the method (approx. 2 ng/ml). In practical terms: minor exposure to ambient smoke may be missed in studies using a urinary cotinine screening for passive smoking of a population. Therefore, we tried

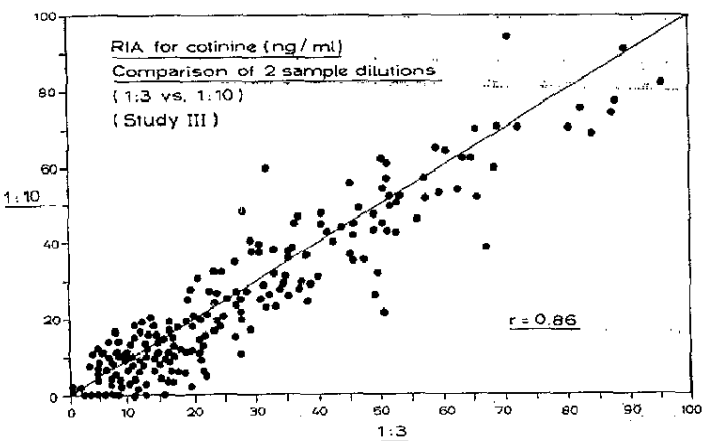

Fig. 2. Variation of urinary cotinine levels between two dilutions of single urine samples (RIA). Values above 100 ng/ml are omitted in this illustration but included for the calculation of the coefficient of correlation.

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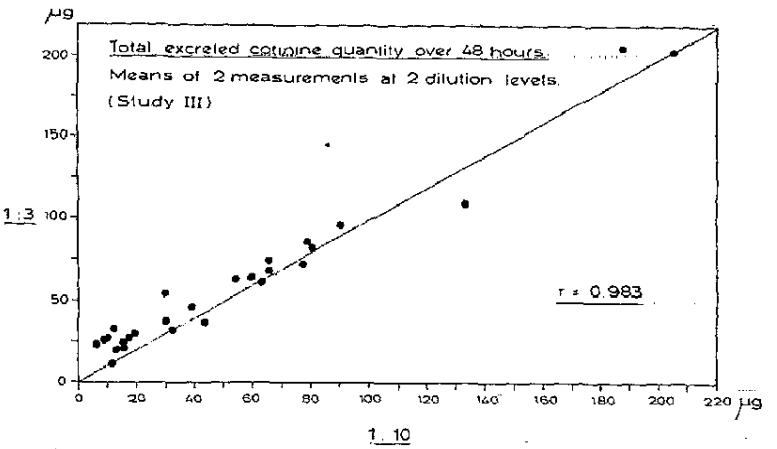

Fig. 3. Variation of two dilutions. Sum of cotinine excretions over 48 h.

to increase the sensitivity of our test during the second study by limiting the dilution to 1:3 (Fig. 2). When comparing the cotinine concentrations in the urine samples of our second study there is an optical tendency to higher levels for the 1:3 dilution. Moreover, considerably more positive samples were obtained at dilution 1:3 than with the higher dilution. Most of these samples without detectable levels at 1:10 were baseline samples obtained before the exposure sessions. Since all participants were known non-smokers who had avoided ETS during the days before the experiment, we conclude that the 1:3 dilution leads to falsely high concentrations caused by more cross reactions. In other terms our findings could mean that an increase in sensitivity of the RIA method is gained at the price of less specificity. Moreover, the cumulated quantity of excreted cotinine over 48 h (Fig. 3) is highly correlated between the two dilutions ($r = 0.983$). There is a tendency to slightly higher recoveries using the 1:3 dilution. Our results indicate that the 1:10 dilution seems to be the better choice.

TABLE III

WITHIN-LABORATORY VARIATION ESTIMATED FROM A SINGLE URINE FRACTION DIVIDED INTO 5 SAMPLES FOR DOUBLE MEASUREMENTS (STUDY II)

Sample	Cotinine in urine (ng/ml)		Mean
	1st measurement	2nd measurement	
1	2.6	4.0	3.3
2	8.4	7.4	7.9
3	4.6	6.9	5.8
4	8.2	3.7	6.0
5	4.3	4.7	4.5
Median	4.6	4.7	5.8

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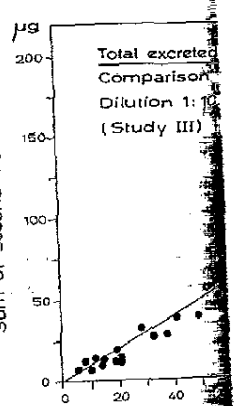

Fig. 4. Variation of urin
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Variation within the laboratory

To obtain information on the variation within the laboratory at low urinary cotinine concentrations we took 5 samples from one urine collection of one person and gave them to one laboratory for double-blind analysis (Table III). The results reported ranged from 2.6 to 8.2 ng/ml (median = 4.65 ng/ml) for the single measurements and from 3.3 to 7.9 ng/ml for the means of each of the 5 pairs. If one divides the total variance of these data into two components – variance between the 5 samples and variance between the 2 measurements of each sample – an analysis of variance reveals that 65% of the total variance can be explained in terms of the variance between the 2 measurements while only 35% is attributable to the variance between the 5 samples. This confirms how important it is to reduce this former variance by using the mean of two measurements. Still, our data indicate that, even when the laboratory techniques are meticulously followed, considerable error variance at low concentrations of cotinine may occur.

This seems to limit the precision with which ETS exposure can be quantified from urinary cotinine levels. This may be true for single measurements but the test-retest reliability of the 2 measurements can be greatly increased by using the total amount of the cotinine mass excreted over the total observation period (e.g., 48 h; Fig. 4). This leads to a correlation coefficient of 0.99 between the two measurements. Since most of the error variance of single measurements is random, pooling double measurements of excreted cotinine mass over an extended period of observation of 48–72 h is the most reliable quantitative indicator of recent ETS exposure.

Variation by freezing the samples

During our second experiment (8, 17 ppm CO over 1 and 2 h) the impact of im-

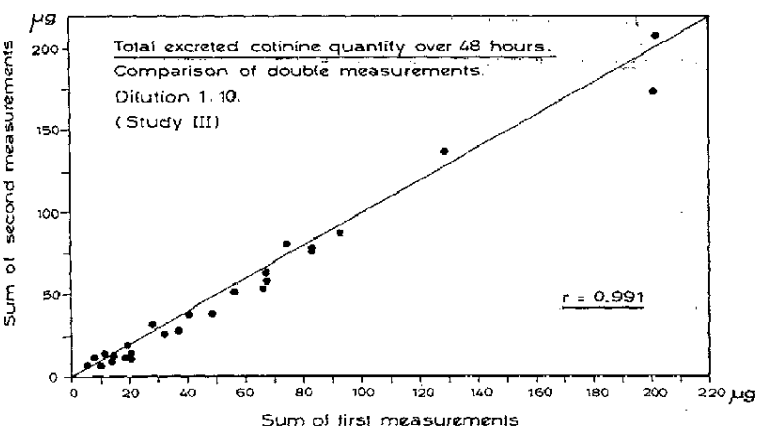

Fig. 4. Variation of urinary cotinine levels within our laboratory. Double measurement of total excreted quantity.

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TABLE IV

MEDIANS OF URINARY COTININE EXCRETION OVER 24 h FOLLOWING POOLED OR FRACTIONED COLLECTION PROCEDURES (STUDY II)

	Urine collection			
	Samplewise (immediately frozen)		Pooled (frozen next day)	
Exposure	8 ppm	17 ppm	8 ppm	17 ppm
1 h (n = 5)	1.7 μg	32.9 μg	0 μg	0 μg
2 h (n = 5)	15 μg	44.9 μg	0 μg	33.7 μg

mediate freezing of the urine samples obtained in the first 24 h after the beginning of the exposure sessions was analysed (Table IV). Every single urine fraction was collected separately. The volume was recorded; 20 ml were immediately frozen. The rest was pooled in a big sampling bottle and frozen the next day. Consistently higher concentrations were measured when the samples were frozen immediately. This could indicate chemical reactions in the sampling bottle. Another explanation is a dilution of urine quantities containing low but just detectable cotinine levels by earlier urine quantities not yet containing cotinine at sufficient concentrations. Such a mechanism could result in a total concentration of the 24-h urine just below detection level. For these reasons we propose that every single urine fraction should be analysed separately.

Total amount of cotinine excreted over up to 72 h

In studies published up to now cotinine concentrations or cotinine/creatinine con-

TABLE V

MEDIANS OF CUMULATIVE COTININE EXCRETION (μg) AS A FUNCTION OF OBSERVATION PERIODS (STUDY II)

Exposition		Cotinine excretion in			
Level	Duration	72 h	24 h	Evening-urine + morning-urine	Morning-urine
8 ppm	1 h	4.3 (100%)	1.7 (39.5%)	1.7 (39.5%)	1.2 (27.9%)
	2 h	33.4 (100%)	15.4 (46.1%)	8.6 (25.7%)	2.6 (7.8%)
17 ppm	1 h	64.6 (100%)	32.9 (50.9%)	19.0 (29.4%)	12.0 (18.5%)
	2 h	79.2 (100%)	44.9 (56.7%)	25.3 (31.9%)	12.0 (15.2%)

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DISCUSSION

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centration ratios were commonly used. These concentrations vary with level and duration of exposure. But being concentrations they cannot be added up over sampling periods. This is one of the reasons why we feel that the total amount of cotinine (volume × concentration) is a more suitable quantitative indicator of actual exposure. However, the amount of cotinine to be found varies not only with level and duration of exposure but also with the length of time over which the urine is collected (Table V). This can easily be shown in some results from our second study. A single sample, e.g. from the first morning after exposure yields only 8%–28% of the amount of cotinine gained over 72 h. During the first 24 h 40% to 56% can be expected. This shows that the precision of urinary cotinine as a quantitative indicator for the exposure to recent ETS can be increased by prolonged sampling periods of from 48 to 72 h.

DISCUSSION

Several investigators [1–4, 5, 10–13] have shown that measurable concentrations of nicotine and cotinine can be found in plasma, saliva and urine after ETS exposure. Plasma levels in our studies were very low even after extreme exposure conditions. Following low exposure plasma may not be sensitive enough. Moreover, plasma samples are more difficult to obtain in epidemiological studies in a population. Finally, the amount of cotinine found in single plasma samples is confounded by the variable time gap between exposure and sampling. This latter argument holds for saliva levels as well.

The measurement of cotinine excretion in urine is currently the best choice. In our first experiment RIA led to higher cotinine levels and was therefore used for the other two studies. Both methods showed considerable variation from various sources, especially after low ETS exposure. In our investigations we tried to identify some of these sources of variation. There is marked variation between laboratories. From the results presented by Biber et al. at this conference [6] we calculated by analysis of variance that about 60% of the total variance of cotinine levels in urine can be explained by interlaboratory variance. This shows that results for different study groups are not easy to compare, even if apparently identical methods are used. Within laboratory variation is also high but can be controlled experimentally by accumulating the total cotinine mass found during continuous urine sampling over a period of 48 to 72 h after exposure and by double measurements. The specific conditions under which the RIA method is conducted influences the actual levels to be found: less dilution of the original samples increases sensitivity at the cost of specificity at low levels. The most stable indicator of recent low-level ETS exposure is to analyse the total urine excreted over a period of 48 to 72 h using a fractional sampling technique. The total amount of cotinine excreted over this period of time is superior to cotinine concentrations and/or cotinine-creatinine ratios found in single urine samples.

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We have shown that without fractioned sampling over 48–72 h there is substantial variation and we recommend, therefore, that this technique should be used in future studies.

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**DETERMINATIC
AND URINE: AN**

(Smokers; non-sm
variation)

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(Received 15 Septemb
(Accepted 25 Septemb

SUMMARY

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*Presented at the Int
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Abbreviations: c.v.,
chromatography; RIA

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